THE PRECIPITIN REACTION BETWEEN TYPE III PNEUMOCOCCUS POLYSACCHARIDE AND HOMOLOGOUS ANTIBODY

III. A Quantitative Study and a Theory of the Reaction Mechanism*

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(Received for publication, December 27, 1934)

In the first paper of this series (1) it was concluded as a first approximation that under a standard set of conditions the entire course of the precipitin reaction between the specific polysaccharide of Type III pneumococcus and homologous purified antibody could be quantitatively accounted for by three simple equations. The mass law was believed to hold for these equations, the more so as the reactions were found to be reversible. Studies of the theoretical factors involved have since been continued under more varied conditions, and the present paper describes experiments which have necessitated modification of the conclusions originally drawn.

EXPERIMENTAL

The quantitative precipitin determinations were made as in previous papers (2–4), except that the technique was modified as described below in order to study the effect of varying a given set of conditions. In general, precipitates were analyzed, rather than supernatants, as had been done in (1). Much of the serum used was obtained through the kindness of Dr. William H. Park, to whom the writers again wish to express their gratitude. Unless otherwise stated, antibody solutions were prepared according to Felton (6). The specific poly-

* The work reported in this communication was carried out under the Harkness Research Fund of the Presbyterian Hospital, New York.

saccharide of Type III pneumococcus is referred to throughout as S III.

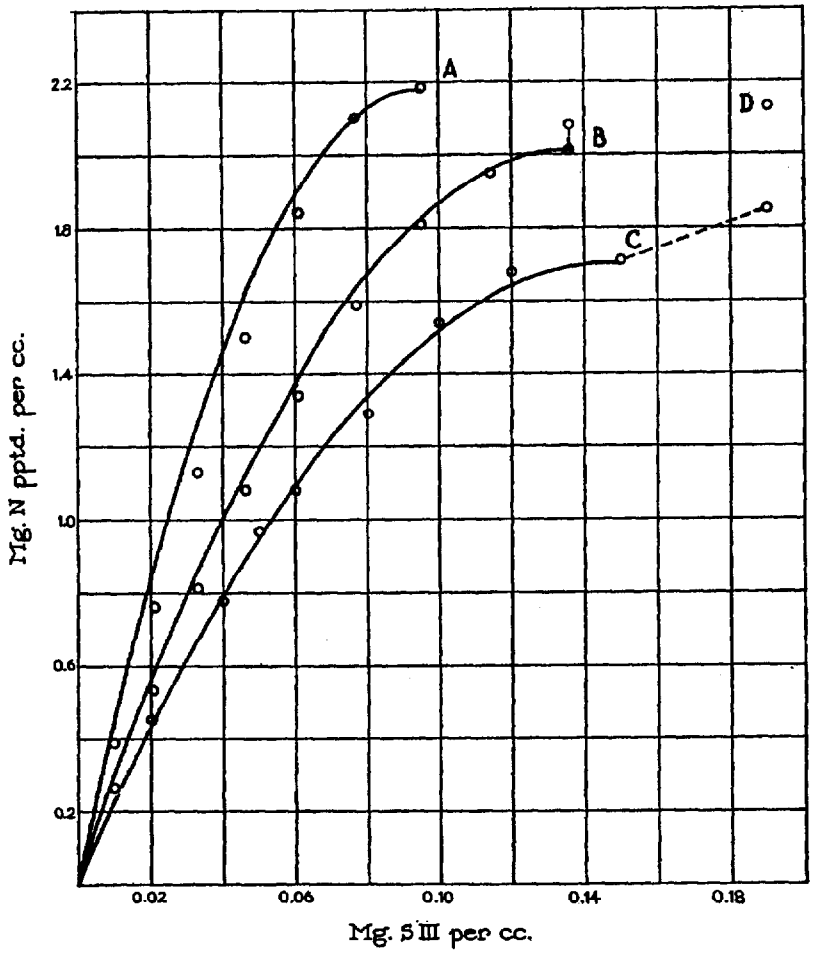
In view of the temperature effects shown in the preceding paper (7) it was found necessary to work at a single temperature in studying the reaction mechanism, and experiments were accordingly run either at 0° or at 37°C.

The differences between the amounts of antibody nitrogen precipitated when the reaction mixture is kept in the cold throughout (0°) and when the precipitation and centrifugation are carried out at 37° are strikingly shown in Tables I and II, which summarize, respectively, the results of the addition of increasing amounts of S III to 1 cc. portions of serum or antibody solution and of serial additions of small amounts of S III to antibody solutions under those conditions.

1 a. Addition of Increasing Amounts of S III to Antibody at 37° and at 0°.—Experiments corresponding to those in the earlier work (1) have now been made with the temperature constant throughout. Duplicate tubes were set up at 37° using 1.0 cc. of antibody Solution B 61 and varying amounts of S III in a total volume of 4 cc. The tubes were incubated for 2 hours at 37° and then centrifuged at that temperature. The precipitate was washed twice with cold saline and analyzed for nitrogen. The results are given in Table I, Column 2, and are represented by the circles along Curve C, Fig. 1. The point connected with the curve by the dotted line indicates the maximum amount of nitrogen specifically precipitable (0.4 mg. S III) from this solution at 37°. A similar experiment was run with antibody Solution B 62 in which the tubes were set up at 0° and centrifuged in the cold instead of at 37°. The amount of S III combined in the region of excess S III was found by determining the amount left in the supernatant by the method described in a previous paper (3), except that the determinations were run at 0° in the B 62 experiments and entirely at 37° in the B 61 series. Aliquot portions of the supernatants containing a suitable amount of S III were set up with another 1.0 cc. portion of the antibody solution used in the experiment, adding saline to bring the volume to 4 cc. The precipitates were analyzed after 24 hours. The amount of S III in the B 61 aliquots was read off from the experimental points along Curve C, Fig. 1 (Column 2, Table I). A similar curve was constructed for B 62. The results are given in Table III.

1 b. Serial Experiments at 0° and at 37°.—In these experiments successive small portions of the antibody were precipitated. In the 0° experiment the reagents were chilled in an ice bath. Duplicate 5.0 cc. portions of antibody Solution B 61 were measured into Wassermann tubes and mixed with 0.5 cc. of a 1 to 10,000 solution of S III. The tubes were kept in the ice box overnight and were then centrifuged in the cold. The supernatants from the duplicate tubes were mixed

and 5.0 cc. samples set up with 0.5 cc. of S III as before. This procedure was repeated until the antibody was exhausted. In the case of antibody Solution



TEXT-FIG. 1

B 62, 0.02 mg. portions of S III were added. The precipitates were washed and analyzed as in the preceding section.

The 37° experiment was carried out in the same way except that the solutions were mixed at room temperature. The tubes were allowed to stand for 2 hours at 37° and were then centrifuged in a small angle centrifuge at 37°.

TABLE I

Addition of Increasing Amounts of S III to 1.0 Cc. of Serum or Antibody

Amount S III added	Antibody Solution B 61, 37°			Horse Serum 607, 0°			Antibody Solution B 62, 0°			Horse Serum 607, 37°			Antibody Solution B 62, 37°		
	N pptd.	N:S III in ppt.	Tests on supernatant	N pptd.	N:S III in ppt.	Tests on supernatant	N pptd.	N:S III in ppt.	Tests on supernatant	N pptd.	N:S III in ppt.	Tests on supernatant	N pptd.	N:S III in ppt.	Tests on supernatant
mg.	mg.			mg.			mg.			mg.			mg.		
0.01							0.36	36.0							
0.02	0.45	22.5	No S, xs A*	0.62	31.0	No S, xs A	0.57	28.5	No S, xs A	0.42	21.0	No S, xs A	0.44	22.0	No S, xs A
0.03							0.78	26.0	" " " "						
0.04	0.79	19.8	" " " "	1.03	25.8	" " " "				0.74	18.5	" " " "			
0.05	0.97	19.4	" " " "				1.07	21.4	" " " "						
0.06	1.08	18.0	" " " "	1.25	20.8	" " " "							0.96	16.0	" " " "
0.075				1.35	18.0	" " " "	1.25	16.7	No S or A	1.16	15.5	" " " "			
0.08	1.29	16.1	" " " "												
0.09				1.40	15.6	" " " "				1.23	13.7	" " " "			
0.10	1.54	15.4	" " " "	1.43†	14.3	No S or A	1.29	12.9	" " " "				1.20†	12.0	S, trace A
0.12	1.68	14.0	" " " "	1.51†	12.6	" " " "									
0.15	1.71	11.4	" " " "	1.59†	10.6	" " " "	1.25		Excess S	1.35†	9.0	No S or A	1.18†		Excess S
0.20	1.75		Excess S							1.32†		Excess S	1.20†		" "
0.25				1.45		Excess S									

* S = S III; A = antibody; xs A = excess antibody.

† Not run in duplicate.

TABLE II

Serial Additions of S III to Antibody Solutions B 61 and B 62

Precipitation No.	B 61, 0.05 mg. S III used				B 62, 0.02 mg. S III used	
	37°		0°		0°	
	Antibody N pptd.	Ratio N : S III in ppt.	Antibody N pptd.	Ratio N : S III in ppt.	Antibody N pptd.	Ratio N : S III in ppt.
	mg.		*mg.*		*mg.*	
1	1.32	26.4	1.94	38.8	0.83*	41.5
2	1.24	24.8	1.70*	34.0	0.82†	41.0
3	1.15	23.0	1.53	30.6	0.72	36.0
4	1.01	20.2	1.39	27.8	0.61	30.5
5	0.88	17.6	1.17‡		0.56	28.0
6	0.77	15.4	0.80‡		0.47	23.5
7	0.63‡		0.23‡		0.43‡	
8	0.37‡		No ppt.		0.21‡	
9	0.14‡				No ppt.	
10	0.15§					

* One determination lost.

† One determination discarded.

‡ The supernatants from these precipitates gave tests for the presence of S III with excess antibody.

§ 5 cc. supernatant from tube 9, allowed to stand in the ice box overnight, gave this additional precipitate. After centrifugation the supernatant showed a negative reaction for antibody with S III.

TABLE III

Determination of N:S III Ratio in Presence of Excess S III

Amount S III	Antibody N pptd.	Fraction of supernatant used for determination of S III content	Antibody N pptd.	S III in aliquot	Total S III in supernatant	S III in original ppt. (by difference)	N : S III in original ppt.
mg.	*mg.*		*mg.*	*mg.*	*mg.*	*mg.*	
Antibody B 62, set up at 0°, centrifuged at 0–10°							
0.50	1.25	1/8	0.74	0.029	0.23	0.27	4.6
1.00	1.19	3/50	1.01	0.044	0.73	0.27	4.4
1.50	1.18	1/20	1.17*	0.059	1.18	0.32	3.7
2.00	1.16	1/40	1.00*	0.044	1.76	0.24	4.8
3.00	1.02	1/50	1.11	0.053	2.65	0.35	2.9
Antibody B 61, set up at 37°, centrifuged at 37°							
0.25	1.75	3/4	0.17	0.007	0.009	0.241	7.3
0.30	1.79	3/4	0.30	0.013	0.017	0.283	6.3
0.35	1.83	3/4	0.48	0.022	0.029	0.321	5.7
0.40	1.85	1/2	0.56	0.027	0.054	0.346	5.3
0.50	1.84	1/4	0.54	0.025	0.100	0.400	4.6
0.75	1.76	1/8	0.89	0.046	0.368	0.382	4.6
1.00	1.76	3/40	0.92	0.048	0.640	0.36	4.9
1.50	1.65	1/20	1.09	0.059	1.18	0.32	5.2

* One analysis discarded.

The values given in the tables represent the mean of the duplicate determinations unless otherwise stated.

The results of the 0° and 37° serial experiments with B 61 are represented by the circles in Fig. 1 along Curves A and B, respectively, calculating each point back to 1.0 cc. volume. The method of calculating the smooth Curves A, B, and C is given in the theoretical part. The point connected with B by the dotted line indicates the additional amount of antibody nitrogen precipitated when the

TABLE IV

Effect of Volume, Final Concentration of Antibody, and Time of Standing

Volume	Antibody B 62 at 0°C.				Antibody B 61 at 37°C.			
	Antibody N pptd. by 0.03 mg. S III in 24 hrs.	Concentration antibody N	Antibody N pptd. by 0.03 mg. S III in 48 hrs.	Concentration antibody N	Antibody N pptd. by 0.05 mg. S III	Concentration antibody N	Antibody N pptd. by 0.10 mg. S III	Concentration antibody N
cc.	*mg.*	*mg. per cc.*	*mg.*	*mg. per cc.*	*mg.*	*mg. per cc.*	*mg.*	*mg. per cc.*
2	0.88	0.21	0.87	0.21				
4	0.84	0.11	0.91	0.10	0.87	0.25	1.15	0.18
6	0.83	0.08	0.87	0.07				
8	0.88	0.05	0.84	0.06	0.87	0.12	1.16	0.09
10	0.84	0.05	0.84	0.05				
12	0.82	0.04	0.87	0.04	0.85	0.08	1.16	0.06

TABLE V

Nitrogen Precipitated from Antibody B 62 by Methylated S III at 0°

Methylated S III	N pptd.	Supernatant tested with		
		Antibody	Methylated S III	S III
mg.	*mg.*			
0.05	0.43	−	++	
0.10	0.60	+	+	
0.15	0.68	+±	±	
0.25	0.74	++	±	++
0.50	0.83	++	−	++

solution, exhausted at 37°, was cooled to 0°. Point D represents the maximum amount of nitrogen specifically precipitable from 1.0 cc. of B 61 (0.5 mg. S III) at 0°.

2. Effect of Changes in Volume and Concentration.—Duplicate portions of 1.0 cc. of antibody Solution B 62 were set up at 0° with 0.03 mg. of S III in volumes of 2, 4, 6, 8, 10, and 12 cc. One set of tubes was allowed to stand for 24 hours, the other for 48 hours in the ice box. The precipitates were centrifuged off in the cold and aliquot portions of the supernatant were analyzed. Duplicate portions of 1.0 cc. of B 61 were also set up with 0.05 and 0.10 mg. of S III, and after 2 hours at

37° the tubes were centrifuged at room temperature. The results are shown in Table IV.

3. Reaction of Pneumococcus III Antibody with Methylated S III.—S III was methylated with methyl sulfate and sodium hydroxide.[1] Solutions of the sodium salt of the methylated product were found to precipitate with homologous horse antipneumococcus serum but not with the corresponding rabbit serum. The failure to precipitate the Type III rabbit antiserum shows that the reaction in horse serum is not due to small amounts of unchanged S III, the more so because addition of traces of unmethylated S III to the methyl S III results in a prompt reaction with rabbit antiserum. Table V shows that the methylated product precipitates 0.83 mg. of nitrogen from 1.0 cc. of antibody Solution B 62 which contains 1.25 mg. of nitrogen precipitable with S III, or 65 per cent of the total antibody.

An antibody solution was made by Felton's method from serum which had been completely absorbed with the methylated S III. This solution contained 1.60 mg. per cc. of antibody nitrogen precipitable by S III and gave no reaction with the methylated product. It also reacted strongly with the partially hydrolyzed S III fractions which have been described in an earlier paper (8) and which did not precipitate with rabbit antisera. It is believed that these findings necessitate the conclusion that more than one antibody to the carbohydrate is present in the original serum or antibody solution.

DISCUSSION

As a result of a new study of the precipitin reaction under definite temperature conditions in the region of excess antibody ratios far higher than in Reference 1 have been found for antibody nitrogen to S III in the precipitate, ranging as high as 40:1, or greater, at 0° and in the presence of a large excess of antibody. The equivalence point[2] ratios, however, remain much the same, averaging 10.8:1.

In a previous paper (1) it was shown that between the equivalence point and the beginning of the inhibition zone there was a wide range in which additional S III caused no change in the amount of nitrogen precipitated. This range, in which S III is present in excess, has now been investigated in greater detail. It has been found that when increasing amounts of S III are added to a fixed quantity of antibody the amount of S III in the precipitate increases to a maximum and then remains almost constant until solution of the precipitate begins. The limiting value of the N : S III ratio in this zone is very close to 5 and is not affected by temperature changes (Table III).

[1] Details of the methylation will be given in a later paper.

[2] Designated equilibrium point in Reference 1.

Thus the ratio of antibody nitrogen to S III varies between more than 40:1 and about 5:1 in the reaction range in which a precipitate is formed. It was also shown in the earlier studies (1) that the soluble compound formed in the inhibition zone contains one more molecule of S III than the immediately preceding insoluble compound. This great variation in composition indicates that antibody and S III are multivalent with respect to each other ((1); also (5, 9, 10), Arrhenius (11), Fleischmann and Michaelis (12), for data on other systems)). The chemical structures of both components of the reaction afford strong support for this view. S III is a polysaccharide built up of at least 10 identical aldobionic acid units joined by glucosidic linkages (13). The group or groups responsible for its reactivity must thus be repeated many times in the molecule. The fact that the partial hydrolysis products react with antibody (8) shows that the reaction is due to certain groupings in the molecule and is not a property of the molecule as a whole. The antibody appears to be a serum globulin of higher molecular weight than normal serum globulin.[3] Since it is built up of amino acid units it, too, offers opportunity for the repetition of the groups involved in the precipitin reaction.

The experiment on the effect of volume upon the amount of antibody precipitated (Table IV) shows that changes in the concentration of antibody in the supernatant have no effect upon the ratio of antibody nitrogen to S III in the precipitate. This apparent contradiction to the requirements of the mass law shows that the explanation of the reaction given in Reference 1 is not adequate. However, the difficulty raised by this finding might with equal justice be urged against attempts such as those of Burnet (14) and Taylor (15) to explain the varying ratios on the basis of adsorption. Thus the Freundlich adsorption equation $\frac{y}{a} = Kx^{\frac{1}{n}}$ states that the amount adsorbed per unit of surface is proportional to some power of the concentration. If, however, one accepts the writers' conclusions, expounded below, regarding the nature of the reaction, one may still use simple chemical equations and apply the mass law, as will be seen in the mathematical treatment in the following section.

One reason for the selection of the S III-antibody system for study

[3] Unpublished diffusion experiments.

was the fact that the S III could be prepared in a pure state. It was felt that one of the components of the reaction was a single substance, and that for this reason the homologous antibody might also be homogeneous. However, evidence has been accumulating which indicates that different parts of a hapten molecule may act independently in stimulating the formation of antibodies and in reacting with them. The cross-reactions between the antibodies formed in response to injection of proteins linked to certain haptens which have been studied extensively by Landsteiner (16) and by Avery, Goebel, and Babers (17) lead to this conclusion (*cf.* also Hooker and Boyd (18)). This is now shown to apply to S III as well. If the hydroxyl groups are covered by methyl groups, leaving the carboxyl groups free, the resulting compound is still reactive with Type III pneumococcus anticarbohydrate. However, it then precipitates only two-thirds of the antibody present (see Table V). Dissociation of the antibody-methyl S III compound does not explain this finding, for the supernatant, after purification and concentration by the Felton method, still failed to precipitate methyl S III. The remainder of the antibody may be precipitated by unmethylated S III, in which the hydroxyl groups are free to react. This indicates not only that more than one kind of antibody is present, but also that on the S III molecule there is, in addition to the molecular grouping carrying hydroxyl groups, at least one other molecular grouping which is immunologically reactive and independently so.

Other observations also show that the antibody is a mixture. In serial experiments after much of the antibody has been removed by successive additions of S III, a point is reached at which an appreciable quantity of S III occurs in the supernatant in the presence of a concentration of antibody which would precipitate S III in a dilution of 1:10,000,000 if the antibody remaining had the same properties as the original antibody. Thus the last portions of antibody to be precipitated appear to show a higher dissociation in the reaction with S III than do the portions which react first.

Again, the difference in the quantity of antibody precipitated at 0° and 37° indicates that a fraction of the antibody forms such soluble or highly dissociated compounds with S III that they cannot be completely precipitated at the higher temperature. The fact that the same amount of antibody is precipitated by S III at 37° from volumes of 4, 8, and 12 cc. (Table IV) shows that the difference is not due to different solubilities of a homogeneous antibody-S III complex at the two

temperatures or to dissociation of such a complex. There is a difference of 0.20 mg. in the amount of nitrogen precipitated when 0.05 mg. of S III and 1.0 cc. of B 61 are set up in a volume of 4 cc. at the two temperatures. The difference should be twice as great in a volume of 8 cc. and three times as great in a volume of 12 cc. if solubility of the precipitate were the cause of this discrepancy, and should be even greater if dissociation of a single compound occurred as well. Thus the serial experiments, the result of carrying out the precipitin reaction at different temperatures, and the experiments with methylated S III all indicate that when horses are immunized with Type III pneumococcus more than one antibody is formed which is reactive with the homologous specific polysaccharide.

SUMMARY

1. In the precipitin reaction between the specific polysaccharide of Type III pneumococcus (S III) and homologous antiserum or purified antibody derived from the horse, the temperature at which the reaction is carried out influences the amount of antibody precipitated.

2. The course of the S III-antibody reaction was studied both at 37° and at 0° from the region of excess antibody to the region of excess hapten. Over the whole range the ratios of antibody nitrogen to S III in the precipitate varied from more than 40:1 to less than 5:1.

3. The amount of antibody nitrogen precipitated under a given set of conditions was found to be uninfluenced by the actual antibody concentration, but to depend on the relative proportions of S III and antibody.

4. This and other evidence is considered to indicate the presence in the antibody solutions and sera of more than one antibody reactive with S III.

5. The significance of the findings is discussed in terms of the multivalence of S III and homologous antibody with respect to each other.

THEORETICAL PART

Although the principal conclusions arrived at in an earlier paper (1) on the mechanism of the precipitin reaction between the specific polysaccharide of Type III pneumococcus (S III) and homologous antibody still appear valid, it has been found that variation of the experimental conditions produces changes of such character that the earlier formulation no longer appears adequate. It is nevertheless possible,

starting from the laws of classical chemistry, to propose a mechanism for the S III-antibody reaction which accounts for the findings, including the Danysz phenomenon. With certain simplifying assumptions this theory permits the formulation of mathematical expressions which quite accurately describe the experimental results and are applicable to unknown sera. A similar mechanism accounts quantitatively for other instances of hapten-antibody and antigen-antibody interaction, as will be shown in subsequent papers.

In the discussion which follows, antibody is considered to be a protein which may be accurately estimated through the determination of nitrogen in the washed specific precipitate (2–4, 7). Pneumococcus anticarbohydrate occurs in the water-insoluble globulin fraction of antipneumococcus horse serum (6) and must be redissolved in the presence of salt. It exists in solution as a globulin-salt complex (for example, Pauli (19)) and it is this complex which is called antibody (A). It is also considered that S III is a definite chemical compound in a state of high purity (13), so that when the extremely delicate test with homologous antibody fails to reveal its presence in the liquid over a specific precipitate it may be assumed that the entire amount added is in the precipitate.

If increasing, small quantities of S III are added to an excess of antibody, decreasing amounts of antibody are found in the supernatant from the specific precipitate, and a point is finally reached at which "equivalent" amounts of S III and antibody are present and only minimal amounts, if any, both of antibody and S III, may be detected in the supernatant. The writers have termed this stage of the precipitin reaction the "equivalence point" (20), and since it is of importance in the discussion which follows and has been made use of in other connections (21) a detailed consideration of the concept is now given.

The location of the equivalence point with any degree of exactness presents both theoretical and experimental difficulties. If increasing amounts of S III are added to antibody a point is eventually reached at which only traces of antibody remain. This would be the actual equivalence point, were it not for at least two factors. One of these is the dissociation of the antibody-S III compound, which varies with the temperature at which the precipitin reaction is carried out and with the individual serum used. A second factor is the ability of the anti-

body-S III compound to combine with more S III in the region of the equivalence point. As the result, when the amount of S III is increased beyond the point at which traces of antibody are still present in the supernatant, a zone ensues in which small amounts of A and S III are present simultaneously, or in which tests for both A and S III are negative. This might be termed the "equivalence zone." It is followed, as the amount of S III is still further increased, by the appearance of S III in the supernatant in excess. This could be taken to mark the end of the equivalence zone, or, if the reaction were being studied in the inverse sense, by addition of increasing amounts of antibody to relatively much S, it would mark the beginning of the equivalence zone from the side of excess S. The midpoint of the equivalence zone would be the actual equivalence point, as nearly as it could be determined.

The extent of the equivalence zone depends on the individual serum studied, and for a given serum, on the experimental conditions used. It also varies with the hapten-antibody or antigen-antibody system studied.

In Column 2 of Table VI are given approximate N : S III ratios at the beginning of the equivalence zone. These were used in making the calculations in Tables VII, VIII, and IX, since the equivalence zone was approached from the region of excess antibody. In Column 4 are given the approximate ratios at the end of the zone, while in the last column are given the mean ratios, or equivalence point ratios. In Fig. 2 is given a graphic representation of the equivalence zone and the reaction on both sides of the zone,[4] taken from data in Tables I and III for antibody Solution B 62 at 0° (Curve A, Point E on Curve C), and B 61 at 37° (Curves B and C). Point D should be at S III = 3.68.

From Table VI it is apparent that the differences in the equivalence points of the individual antibody solutions lie outside even the large experimental error involved in their determination, and this is characteristic of other immune systems as well. It is, therefore, scarcely

[4] The breadth of the zone in some instances may explain the failure of the "optimal proportions" method to yield the same end-point when antigen is diluted as when antibody is diluted, since the equivalence zone would be approached from a different side in each instance.

possible as was formerly thought, to consider each hapten-antibody, or antigen-antibody system as characterized by a definite equivalence point, although a fairly characteristic average may be found for each system.

Another basis for the discussion which follows is the finding that the ratio of antibody nitrogen to S III in the precipitate depends on the relative proportions of S and A present, and not on their final concentrations. The difficulties raised by the finding that the antibody is a mixture of substances with different reactivities toward S III are

TABLE VI

N:S III Ratios of Various Antibody Solutions

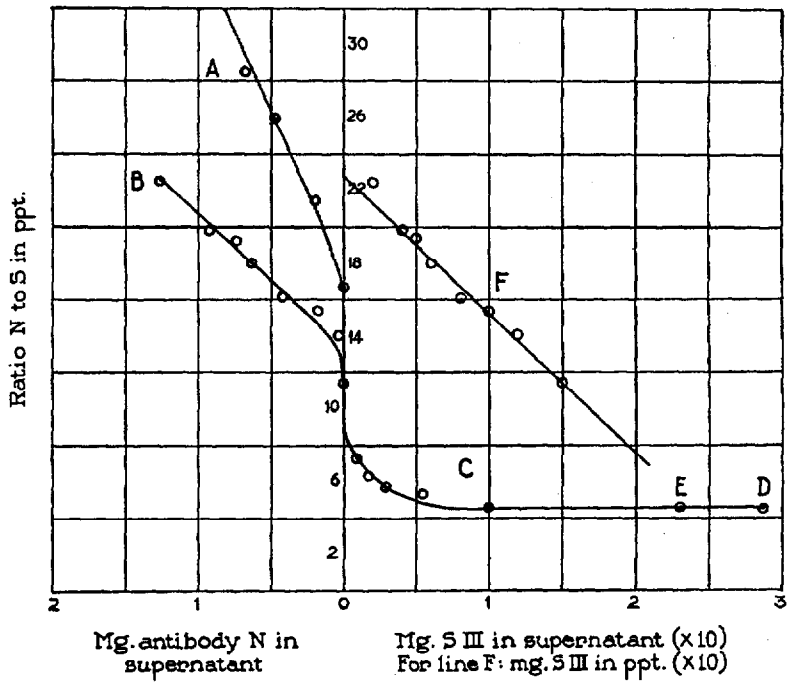
Serum or antibody solution	Ratio at beginning of equivalence zone	Calc. ratio at beginning of equivalence zone	Ratio at end of equivalence zone	Mean ratio (equivalence point)
B III		11.5		11.1
B IV		7.9		8.7
B V_A	13.6	10.5	8.6	(11.1)
B VII		10.8		12.2
B X	(15)	12.5	11.9	(13.5)
B 32				8.7
B 36	12.4	12.4	9.4	(10.9)
B 51	14.1	15.5	(7)	(10.6)
B 60	13.7	13.8	8.4	10.5
B 61, 37°	11.4	11.4	8.8	(10.1)
B 62, 0°	(17)	16.6	8.3	(12.7)
B 62, 37°	12	12.5	7.9	(10)
Serum 607, 0°	(15)	15.3	(8)	(11.5)
Serum 607, 37°	(11)	11.3	(8)	(9.5)
Mean equivalence point ratio.....				10.8

Values in parentheses indicate most probable value deduced from nearest actual determination.

met by assuming that the average behavior of the antibody is that of a single substance; that is, that it behaves statistically as if it were homogeneous. This necessarily results in a more or less artificial structure, but it appears to fit the facts and to be applicable to antigen-antibody systems in general. It is, therefore, offered as an expedient until such time as it may be possible to separate from the complex antibody mixture an antibody possessed of a single chemical reactivity.

Moreover, in the discussion which follows, the multivalence of S III and antibody with respect to each other is an essential premise. It

was shown in the preceding sections that ratios of precipitated antibody nitrogen to S III in the region of excess antibody might be greater than 40:1, while in the region of excess S III the ratio might be less than 5:1. It was also shown previously (1) that the soluble compound formed in the inhibition zone contains one more molecule of S III than the immediately preceding insoluble compound, and would thus be characterized by an even lower ratio. There is thus approxi-



TEXT-FIG. 2

mately a tenfold difference in the extremes of the ratios in which S III and antibody may combine with each other.

A simple explanation of these varying ratios could be based on the observations that more than one antibody and more than one reactive grouping on the S III molecule are involved in the reaction. Thus S III might be considered as having Groups a, b, c, and d which react with the corresponding Antibodies a', b', c', and d'. If the antibodies are present in different proportions the first compound might be S-a', b', c', d'; then after d', the antibody present in smallest amount, is used up,

the precipitate formed would be S-a', b', c', and later S-a', b' and S-a' would be formed. This would account for the changing ratios but it does not explain the Danysz phenomenon, since the same amount of antibody would be precipitated by a given amount of S III regardless of whether all of the S III were added in one portion or in several. That this is not the case is illustrated in Fig. 1, in which Curve C shows the amount of antibody nitrogen precipitated from 1.0 cc. of antibody solution by the addition of various quantities of S III, while Curve B shows the amount of nitrogen precipitated in a serial experiment by the same quantities of S III.[5] It will be seen that all of the antibody is precipitated by a smaller amount of S III in the serial experiment than in the other.

In order to explain the varying ratio of antibody nitrogen to S III in the precipitate, as well as the Danysz phenomenon,[6] it is postulated that at the equivalence point the compound AS (or, more exactly, A_xS_y) is formed. In the region of excess antibody A_2S, A_3S, A_4S, ...A_mS (or, more exactly, $A_{mx}S_y$) may exist, depending on the relative excess of antibody. With excess S III, AS_2 can be formed as an insoluble compound and AS_3 (or, more exactly, A_xS_{3y}) as a soluble compound.

The application of the mass law to this reaction presents difficulties. If the compounds formed are considered to be A_4S, A_3S, A_2S, and AS (at the equivalence point), then as increasing amounts of S III are added to a fixed amount of antibody solution, the compound A_4S should be formed until the concentration of A is reduced to a point at which A_3S would begin to form. Throughout this range the ratio would be constant. Then on addition of more S III all of the precipitate would be converted into A_3S before any change in antibody concentration would take place. In this range the ratio would change, but the amount of antibody nitrogen precipitated would remain constant. Thus the reaction would proceed in a series of steps instead of in the continuous curve shown in Fig. 1. However, if the compounds formed are $A_{4x}S_y$, $A_{3x}S_y$, etc., intermediate steps $A_{4x-1}S_y$ $A_{4x-2}S_y$.....$A_{3x}S_y$ could occur and the steps be brought so close together that a continuous curve would result. In any case, the particular compound formed would be fixed by the concentration of antibody in the supernatant.

However, it has been shown that changes in the concentration of antibody in the supernatant have no effect on the ratio of antibody nitrogen to S III in the precipitate. If the mass law is to be applied it is therefore necessary to find some other basis for its use. Such a

[5] This curve was obtained by calculating each stage back to 1.0 cc. of antibody.

[6] Danysz himself accounted for the effect which he discovered on the basis of the union of antigen and antibody in more than one proportion (22), but his explanation was not acceptable at the time (*cf.* 20).

basis is found if the S III-antibody reaction is considered as a series of successive bimolecular reactions which take place before precipitation occurs. The assumption that the reactions are bimolecular appears reasonable, for studies in all fields of chemistry have shown that more complex reactions are extremely rare. On this basis, then, the first step in the reaction between A and S III would be the formation of the compound AS according to the equation:

$$A + S \rightleftarrows AS \quad [1]$$

In order to simplify the mathematical treatment, the subscripts x and y are dropped and the equivalence point compound is assumed to be AS. That this procedure has only a small influence on the final result is shown later. As both S III and antibody are multivalent with respect to each other the AS compound formed in this reaction could react with other molecules of the same compound, or with S III or A, whichever is present in excess.

Let us consider the subsequent course of the reaction in the region of excess antibody. The second step would consist of the two competing bimolecular reactions:

$$\left.\begin{aligned} AS + A &\rightleftarrows AS\cdot A, \text{ and} \\ AS + AS &\rightleftarrows AS\cdot AS. \end{aligned}\right\} \quad [2]$$

There would follow a third step, in which the competing bimolecular reactions involved would be

$$\left.\begin{aligned} AS\cdot A + A &\rightleftarrows AS\cdot A\cdot A, \\ AS\cdot AS + A &\rightleftarrows AS\cdot AS\cdot A, \\ AS\cdot A + AS\cdot A &\rightleftarrows ASA\cdot ASA, \\ AS\cdot A + AS\cdot AS &\rightleftarrows ASA\cdot AS\cdot AS, \text{ and} \\ AS\cdot AS + AS\cdot AS &\rightleftarrows ASAS\cdot ASAS, \end{aligned}\right\} \quad [3]$$

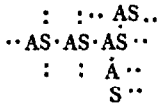
in which the first two reactions would occur only in the presence of enough A to carry the composition of the reaction product beyond the A_2S stage. Similarly, each compound formed in the third step would react with each other compound, or with more A, if present, to form still more complex substances, and the reaction would continue until particles would be formed large enough to settle from the solution and precipitation would take place.[7]

[7] Specific precipitates have been included among the "symplexes" (Willstätter, R., and Rohdewald, M., *Z. Physiol. Chem.*, 1934, **225**, 103).

If A and S III are mixed in equivalent proportions the AS formed in reaction [1] would merely polymerize in steps [2], [3]....., and the equivalence point precipitate would be $(AS)_n$.

In the region of excess S III a similar series of expressions would apply, in which S and A would be interchanged in [2], [3],..... In the presence of a large excess of S, in other words, in the inhibition zone, there would also be present in solution a soluble compound, AS_s, containing one more molecule of S in combination than the last insoluble compound (1). Since this is formed only with a very large excess of S, all of the specific groupings of A would tend to react with S rather than with AS complexes and there would be no large, insoluble, intermolecular aggregates formed.

The final precipitate, then, would in each case consist of antibody molecules held together by S III molecules,



a view similar to that presented recently by Marrack (23) but, it is believed, more definite and more easily treated quantitatively. The process of aggregation as well as the initial hapten-antibody combination is considered to be a chemical reaction between definite molecular groupings. On this basis it is unnecessary to make assumptions as to the change of so called hydrophilic groups into hydrophobic[8] groups, as the process of aggregation would occur regardless of the affinity of the groupings for water.

It is believed that the compounds formed in the first stages of the reaction are soluble. Indeed, in the reaction between antibody and a hapten (H) containing only one reactive grouping, compounds of the type AH_s would be the only ones formed and there would be no opportunity for the building up of aggregates large enough to precipitate. However, with the specific grouping repeated two or more times, as in azo dyes studied by Landsteiner and van der Scheer

[8] The combination of the polysaccharide S III with the antibody would increase the number of "hydrophilic groups" on the molecule rather than decrease them, so that Eagle's explanation for the insolubility of the precipitate would not be applicable (24).

(25), compounds of the type AH·AH..... could be formed and the precipitation actually observed is accounted for. Marrack and Smith have already suggested the necessity of the presence of more than one specific grouping in a hapten in order that specific precipitation may occur (26, 23).

The mathematical treatment of the entire course of the reaction involves certain simplifying assumptions, some of which have already been discussed. It is assumed, first, that the antibody mixture may be treated statistically as though it were homogeneous; second, that in the initial stage of the reaction A reacts with S to give only AS; third, that in the second step of the reaction the products are AS·A and AS·AS; fourth, that the mass law applies, so that the rates of formation of AS·A and AS·AS are proportional to the concentrations of the reacting substances; and fifth, that the dissociation of AS·A and AS·AS is negligible. Although there is no reason to assume discontinuities in the building up of the final aggregates, the reactions are arbitrarily treated as successive stages in order to simplify the mathematics involved.

At the beginning of the second stage of the reaction, then, in the presence of excess antibody,

let A = total units of antibody in the reacting system,
B = units of AS formed in the first step = units of S added,
$A - B$ = units of free antibody at end of first step,
x = units of AS·A formed at time t,
y = units of AS·AS formed at time t, and
V = volume.

Then $\frac{A - B - x}{V}$ = concentration of free antibody at time t, and

$\frac{B - x - 2y}{V}$ = concentration of AS at time t.

Rate of formation of AS·A $= \frac{dx}{dt} = K\left(\frac{A - B - x}{V}\right)\left(\frac{B - x - 2y}{V}\right)$ .....[4]

Rate of formation of AS·AS $= \frac{dy}{dt} = K'\left(\frac{B - x - 2y}{V}\right)^2$ .....[5]

Dividing [4] by [5], $\frac{dx}{dy} = \frac{A - B - x}{B - x - 2y}$ if $K = K'$.

Integrating, $\frac{A - 2x}{2(A - B - x)^2} = \frac{y}{(A - B - x)^2} + C$

To evaluate C: at start of reaction when $t = 0$, $x = 0$, $y = 0$, $C = \frac{A}{2(A - B)^2}$

At the end of the reaction $x + 2y = B$. Therefore,

$$x = \frac{AB - B^2}{A} \text{ and } y = \frac{B^2}{2A}$$

Since each unit of x and y contains 2 units of antibody the number of units of antibody precipitated is given by $2(x + y)$ which equals

$$2B - \frac{B^2}{A} \quad [6]$$

It will be noted that the volume factors cancel, so that the amount of antibody precipitated depends only on the relative amounts of antibody and S III present and not on their concentration.

This treatment of the problem involves only the formation of compounds having ratios between R and 2 R, where R is the ratio of antibody to S III in the equivalence point compound. The experimental data show that compounds having ratios greater than 2 R may be formed, for at 0° in the presence of a large excess of antibody ratios greater than 4 R are encountered. By extending the process used for the calculation of the second step to stage [3] and beyond, it is possible to calculate the amount of antibody precipitated by a given amount of S III when the ratio varies between R and 3 R and also between R and 4 R.

The calculations are complicated, as step [3] involves the bimolecular formation of five compounds, that is, A_3S, A_3S_2, A_4S_2, A_4S_3, and A_4S_4, and extension of the process to 4 R results in 20 compounds. In this calculation it is assumed that the ratio in which any two products are formed is unaffected by the other competing reactions. The expression thus calculated for antibody precipitated in the range R to 3 R is:

$$\text{Units of antibody pptd.} = A - \frac{2(A - B)^4}{A[(A - B)^3 + A^3]}* \quad [7]$$

* Copies of the derivation will be furnished on request.

The same formulas apply in the region of excess S, and in their derivation S and A are interchanged.

In the above calculations the simplifying assumption was made that the composition of the precipitate at the equivalence point is represented by the molecular formula AS. It will now be shown that this assumption is not necessary, and that if the antibody nitrogen: S III ratio in the precipitate varies between the value found at the equivalence point and one twice as great when a large excess of antibody is present, the reaction follows the same course regardless of the molecular composition at the equivalence point.

If the compound at this point be taken as AS_n, formed as a result of a series of bimolecular reactions between A and S, making up the first step of the reaction, the

course of the reaction as far as A_2S_n, which has double the N:S ratio, proceeds similarly to [2]:

$$\left.\begin{aligned} AS_n + A &\rightleftarrows A_2S_n \\ AS_n + AS_n &\rightleftarrows AS_n \cdot AS_n \end{aligned}\right\} \quad [8]$$

In the presence of still more antibody it would proceed according to [3], and both reactions would be calculated as were these steps.

If, on the other hand, the equivalence point compound be taken as A_2S, the course of the reaction between A_2S and A_4S requires two successive steps, as in [2] and [3]:

$$\left.\begin{aligned} A_2S + A &\rightleftarrows A_3S \\ A_2S + A_2S &\rightleftarrows A_2S \cdot A_2S \end{aligned}\right\} \quad [9]$$

and

$$\left.\begin{aligned} A_3S + A &\rightleftarrows A_4S \\ A_3S \cdot A_3S + A &\rightleftarrows A_4S_2 \cdot A \\ A_3S \cdot + A_3S &\rightleftarrows A_3S \cdot A_3S \\ A_4S + A_4S_2 &\rightleftarrows A_3S \cdot A_4S_2 \\ A_4S_2 + A_4S_2 &\rightleftarrows A_4S_2 \cdot A_4S_2 \end{aligned}\right\} \quad [10]$$

and is therefore calculated in the same way as the reaction between AS and A_3S. The expression derived for the reaction between the limits A_2S and A_4S is:

$$\text{Units antibody N pptd.} = A - \frac{2(A - 2B)^4}{(A - B)\,[(A - B)^2 + (A - 2B)^2]}^* \quad [11]$$

* Copies of the derivation will be furnished on request. In Table VII, Columns 1, 2, and 3, a calculation of the reaction is given according to [1] and [2], and [9] and [10], respectively, and it is evident that the differences are small.

In making this calculation and in applying the derived equations to the experimental data it is necessary to convert units of antibody and S III into milligrams. This may be done by assuming that 1 mg. of antibody nitrogen equals 1 unit, that the number of milligrams of antibody nitrogen precipitated at the equivalence point equals A, and that the ratio of A to S III at this point is equal to R. It follows that in equations [6] and [7] B = A and B = RS at the equivalence point. Equation [6] then becomes:

$$\text{mg. antibody N pptd.} = 2\,RS - \frac{R^2S^2}{A} \quad [6a]$$

Equation [7] becomes

$$\text{mg. antibody N pptd.} = A - \frac{2(A - RS)^4}{A[(A - RS)^2 + A^2]} \quad [7a].$$

and since in equation [11] 2 B = A at the equivalence point and 2 B = RS, this equation becomes:

$$\text{mg. antibody N pptd.} = A - \frac{2(A - RS)^4}{\left(A - \frac{RS}{2}\right)\left[\left(A - \frac{RS}{2}\right)^2 + (A - RS)^2\right]} \quad [11a]$$

In order to permit comparisons to be made between antibody solutions containing different amounts of antibody and having different equivalence point ratios it was found convenient to reduce the amounts of S III and N precipitated to percentages of the quantities precipitated at the equivalence point. To convert [6 *a*] into an expression involving percentages, use is made of the relationship $A = RS_{eq.}$ at the equivalence point. Dividing all terms of [6 *a*] by $RS_{eq.}$

$$\frac{\text{N pptd.}}{RS_{eq.}} = 2\frac{RS}{RS_{eq.}} - \frac{\frac{(RS)^2}{(RS_{eq.})^2}}{\frac{A}{RS_{eq.}}} = \frac{\text{N pptd.}}{A} = 2\frac{S}{S_{eq.}} - \frac{\frac{S^2}{S_{eq.}^2}}{\frac{A}{A}}$$

Multiplying each side of the equation by 100,

$$\text{Per cent A pptd.} = 2 \times \%S - \frac{\%S^2}{100} \quad \ldots\ldots\ldots\ldots\ldots [6b]$$

$$\text{Per cent A pptd.} = 100 - \frac{2(100 - \%S)^4}{100\,[(100 - \%S)^2 + 100^2]} \quad \ldots\ldots\ldots [7b]$$

$$\text{Per cent A pptd.} = 100 - \frac{2(100 - \%S)^4}{\left(100 - \frac{\%S}{2}\right)\left[\left(100 - \frac{\%S}{2}\right)^2 + (100 - \%S)^2\right]} \quad [11b]$$

The percentages of A precipitated by increasing percentages of S III, calculated according to these equations, are given in Table VII. These data are shown graphically in Fig. 3, in which Curve A is calculated according to [7 *b*] and Curve B according to [6 *b*].

In Table VIII the data on Serum 607 in Table I are calculated in terms of percentage of the total precipitated at the equivalence point, and the percentage of S III used and antibody N precipitated are plotted on Fig. 3. It will be seen that the circles representing the values found at 0° lie very close to the curve (A) for the reaction in which the N : S ratio varies from R to 3 R (equation [7 *b*]) while the 37° values are very near those calculated for the R to 2R reaction (equation [6 *b*], Curve B). Thus the course of the reaction at 0° appears to be determined by a greater complexity of the reactions occurring after the initial A and S combination than is indicated by the data for the reaction at 37°.

If, instead of using the equivalence point as the basis of the calculation, the ratio at the beginning of the equivalence zone (from the region of excess antibody) be used, the course of the reaction follows the two stage expression [6 *a*] very closely in all but one of the anti-

TABLE VII

Calculated Percentage of Antibody Precipitated

Total S III added	Antibody N precipitated		
Calculated according to.....	Equation 6 *b*	Equation 11 *b*	Equation 7 *b*
Ratio limits.....	R and 2 R	R and 2 R	R and 3 R
per cent	*per cent*	*per cent*	*per cent*
10	19.0	19.4	27.5
20	36.0	37.2	50.0
30	51.0	53.4	67.8
40	64.0	67.6	80.9
50	75.0	79.5	90.0
60	84.0	88.7	95.6
70	91.0	95.1	98.5
80	96.0	98.7	99.7
90	99.0	99.88	99.98
100	100.0	100.0	100.0

TABLE VIII

Antibody N Precipitated by S III from Serum 607
Expressed as Percentage of Quantity Precipitated at Equivalence Point

S III used	Reaction at 0°			Reaction at 37°		
	S III	Antibody N pptd.	Antibody N pptd.	S III	Antibody N pptd.	Antibody N pptd.
mg.	*per cent*	*mg.*	*per cent*	*per cent*	*mg.*	*per cent*
0.02	14.5	0.62	42.8	15.8	0.42	31.6
0.04	29.0	1.03	71.0	31.5	0.74	55.6
0.06	43.5	1.25	86.2			
0.075	54.4	1.35	93.1	59.1	1.16	87.2
0.09	65.2	1.40	96.6	70.9	1.23	92.5
0.10	72.5	1.43	98.6			

body solutions studied in sufficient detail, regardless of the temperature at which the reaction is carried out.[9] The theoretical amounts

[9] The exception, BX, is one of the solutions studied in the beginning of the work (1), in which aliquot portions of supernatant were analyzed instead of entire precipitates. It was, moreover, an exceedingly concentrated solution.

of antibody nitrogen precipitated by varying quantities of S III from different antibody solutions according to equation [6 *a*] were calculated with the aid of the experimental values for R given in Table VI for the ratio at the beginning of the equivalence zone, A being nitrogen precipitated at this point. A comparison is given in Table IX of the calculated and experimental values for nitrogen precipitated.

In a previous paper (3) it was shown that the antibody nitrogen precipitated by S III from Solution B 31 in the region of excess antibody followed the empirical equation, $N = 18.6\,S - 60\,S^2$. It will be noted that this equation is in the same form as [6 *a*], so that the theoretical significance of the two constants is now clear, for $18.6 = 2R$ and $60 = \frac{R^2}{A}$.

The results of the serial experiments (Table II) also conform to equation [6]. In order to make the comparison with other data, the result from each successive addition of S III was calculated to the 1.0 cc. basis. The ratio of total antibody nitrogen precipitated to total S III used was taken as R and the total antibody nitrogen per 1.0 cc. of antibody solution as A in the equation [6*a*]. Curves A and B, Fig. 1, were calculated with the aid of these values. The circles along the curves represent the actual experimental data.

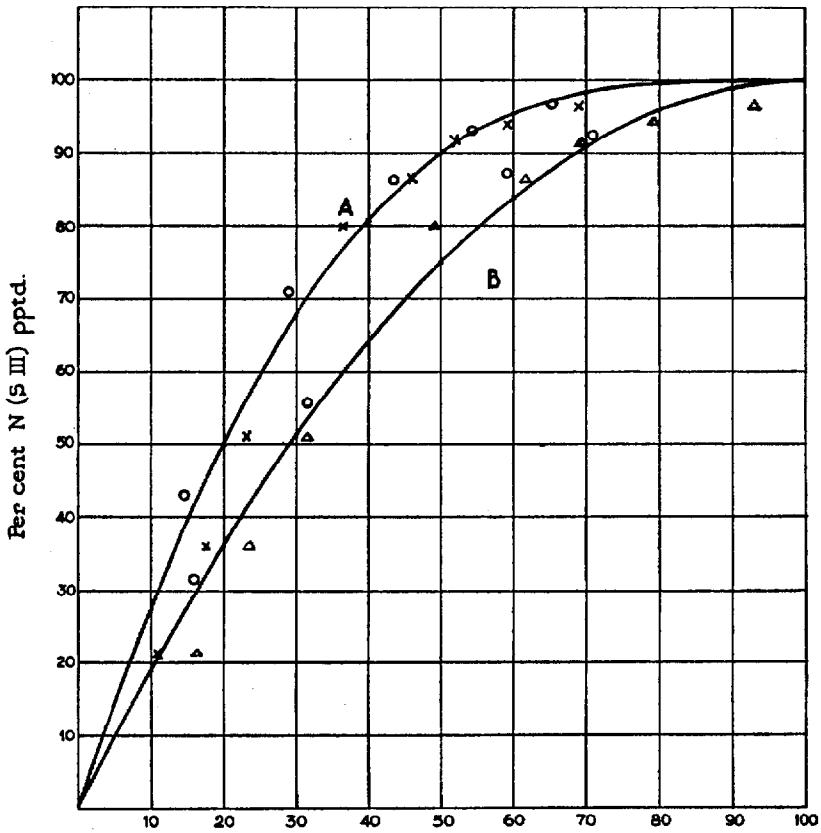
In the region of excess S III, by interchange of S and A (page 581), equation [6 *a*] becomes:

$$\text{mg. S III precipitated} = 2\,R'A - \frac{(R')^2A^2}{\text{Total S}} \quad \ldots\ldots\ldots\ldots\ldots [12],$$

in which $R' = \frac{S}{A}$ at the equivalence point.

Thus, as in equation [6], the amount of S precipitated depends only on the relative amounts of S and A present. Expressions similar to [12] may also be found corresponding to [6*b*], [7*a*], and [7*b*] by interchanging A and S. In making calculations according to these equations 100 per cent A was taken as $\frac{\text{Total S III present}}{R'}$, or the amount which would combine with the S III present to form the equivalence point compound. The points obtained from the data in Table III are plotted as crosses in Fig. 3, using the per cent of S III pre-

cipitated as ordinates and the per cent of A as abscissae. It will be seen that the values fall quite close to the theoretical values for a three step reaction. If, however, instead of the ratio of N : S at the



TEXT-FIG. 3

equivalence point, the ratio be taken as 7.5, at which point an analytically determinable amount of S III first appears in the supernatant, the reaction follows the two stage mechanism, according to equation [6 *b*]. This procedure is analogous to that used in the region of

excess antibody. The points derived in this way are plotted as triangles in Fig. 3. The three lowest points are partially in the inhibition zone and could scarcely be expected to conform closely to the curves. Similar considerations apply to antibody Solution B 62 at 0°, for which the data are also given in Table III.

TABLE IX

Comparison of Experimental Data with Values Calculated According to:

$$N \text{ Precipitated} = 2RS - \frac{R^2S^2}{A}$$

Antibody No.....	BV_A		B 36		B 61		B 62		B 62		Serum 607		Serum 607	
Temperature, °C..	37, 0		37, 0		37, 37		0, 0		37, 37		0, 0		37, 37	
R.....	13.6		12.4		11.4		(17)		12		(15)		(11)	
A.....	4.08		1.86		1.71		(1.23)		1.20		(1.42)		(1.31)	
	N pptd.		N pptd.		N pptd.		N pptd.		N pptd.		N pptd.		N pptd.	
S III used	Found	Calc.	Found	Calc.	Found	Calc.	Found	Calc.	Found	Calc.	Found	Calc.	Found	Calc.
mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
0.01							0.36	0.32						
0.02			0.50	0.46	0.45	0.43	0.57	0.59	0.44	0.43	0.62	0.54	0.42	0.40
0.03							0.78	0.81						
0.04					0.79	0.79					1.03	0.95	0.74	0.73
0.05	1.22	1.25	1.03	1.03	0.97	0.95	1.07	1.11						
0.06					1.08	1.09			0.96	1.01	1.25	1.23		
0.075			1.41	1.40							1.35	1.36	1.16	1.13
0.08					1.29	1.34								
0.09											1.40	1.42	1.23	1.23
0.10	2.24	2.27	1.66	1.65	1.54	1.52								
0.12					1.68	1.64								
0.20	3.62	3.62												
0.25	3.87	3.96												

R and A values in parentheses deduced from nearest actual determination.

A relationship useful in its application to unknown sera may be derived from expression [6 *a*]. If both sides of the equation be divided by S, the resulting equation, $\frac{N}{S} = 2R - \frac{R^2}{A}S$, is that of a straight line. Thus, if the values of the ratio found in the region of excess antibody are plotted as ordinates against the amounts of S III added as abscissae, a straight line is obtained. The intercept on the

y axis gives the value of 2R, while the slope is $\frac{R^2}{A}$, from which A, the amount of nitrogen precipitated at the beginning of the equivalence zone, may be calculated. Line F, Fig. 2 (page 576), illustrates this

TABLE X

Calculation of Precipitated Antibody N from N:S III Ratio at Two Points by Means of Linear Relation $\frac{N}{S} = 2R - \frac{R^2}{A}S$

Antibody No.	BV_A		B 36		B 61		B 62		Serum 607		Serum 607	
Temperature, °C.	37, 0		37, 0		37, 37		0, 0		0, 0		37, 37	
S III added at 2 points used, *mg.*	0.10, 0.20*		0.05, 0.10*		0.05, 0.10*		0.03, 0.05*		0.04, 0.075*		0.04, 0.075*	
Values given by line drawn through the 2 points: 2R	26.8		24.6		23.4		33		34.8		21.9	
Values given by line drawn through the 2 points: $\frac{R^2}{A}$	43.5		80		80		233		224		85.5	
	N pptd.		N pptd.		N pptd.		N pptd.		N pptd.		N pptd.	
	Found	Calc.	Found	Calc.	Found	Calc.	Found	Calc.	Found	Calc.	Found	Calc.
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
A	4.08	4.13	1.86	1.89	1.71	1.71		1.17		1.35		1.40
Amt. S III *mg.*												
0.01							0.36	0.31				
0.02			0.50	0.46	0.45	0.44	0.57	0.57	0.62	0.61	0.42	0.40
0.03							0.78*					
0.04					0.79	0.81			1.03*		0.74*	
0.05	1.22	1.23	1.03*		0.97*		1.07*					
0.06					1.08	1.12			1.25	1.28		
0.075			1.41	1.40					1.35*		1.16*	
0.08					1.29	1.36						
0.09									1.40	1.32	1.23	1.28
0.10	2.24*		1.66*		1.54*							
0.12					1.68	1.66						
0.20	3.62*											
0.25	3.87	3.98										

* These points are also marked with an asterisk in the N pptd. columns below.

procedure in the case of antibody Solution B 61, the circles being the experimentally found ratios in Column 3, Table I. The calculated ratios at the beginning of the equivalence zone given in Column

3 of Table VI were obtained in this way and are probably more accurate than the observed ratios because the experimental errors in the determination of the individual points are averaged in this method.

This linear relationship makes it possible to characterize an unknown Type III antipneumococcus serum or antibody solution in the region of excess antibody by two analyses, in duplicate. If the ratio of antibody N to S III precipitated be determined for two different amounts of S III in the region of excess antibody and a straight line be drawn through the two points so obtained, the intercept on the y axis = 2 R and the slope = $\frac{R^2}{A}$. With the R and A values at the beginning of the equivalence zone calculated in this way the amount of antibody nitrogen precipitated by any quantity of S III less than $\frac{A}{R}$ may be calculated with a fair degree of accuracy. In choosing the amounts of S III to be used in the determination of these points it is best to precipitate more than 50 per cent of the antibody, since above this level experimental errors in the determination of nitrogen have a smaller effect on the $\frac{N}{S}$ ratio. The application of this procedure to several antibody solutions is illustrated in Table X. It will be seen that there is in general good agreement between the observed and calculated points, but it is better, of course, to have three points with which to determine the position of the line.

In Fig. 2 and in making calculations in the region of excess antibody it is assumed that all of the antibody present is precipitated at the beginning of the equivalence zone. The data in Reference 1 and in Tables I and III show that this is actually not the case, and that the amount of antibody precipitated usually increases as S III is increased in the equivalence zone, often reaching its maximum only when S III is present in appreciable excess. In different sera the amount of additional antibody nitrogen precipitated in this way varies from a few hundredths to one- or two-tenths of a milligram. This behavior appears due to the varying amounts of the relatively easily dissociable antibody occurring in different sera, and renders necessary for the complete description of the behavior of a serum in the precipitin reaction a separate determination of the maximum amount of specifically precipitable nitrogen (3, 4, 7).

In the region of excess S III the behavior of a serum as far as the beginning of the inhibition zone may be characterized by the determination of the A and S III precipitated at two points, since in this region the linear relation $\frac{\text{S pptd.}}{\text{A}} = 2\text{R}' - \frac{(\text{R}')^2\,\text{A}}{\text{Total S}}$ applies if R′ be taken as the $\frac{\text{S}}{\text{A}}$ ratio at the end of the equivalence zone at which S III appears in excess and A be taken as the amount of antibody precipitated.

In the inhibition zone, in which large amounts of S III are present and the amount of precipitate has begun to diminish, this equation is no longer applicable and it is necessary to determine the dissociation constant of the soluble compound AS_n according to the method indicated in Table V, Reference 1. The determination of two properly spaced points should be sufficient to establish the dissociation constant and permit the calculation of other points in this range.

SUMMARY AND CONCLUSION

The precipitin reaction between the specific polysaccharide of Type III pneumococcus and homologous antibody formed in the horse can be accounted for quantitatively by assuming the chemical combination of the components in a bimolecular reaction, followed by a series of competing bimolecular reactions which depend upon the relative proportions of the components. These reactions would lead to the formation of larger and larger aggregates until precipitation ultimately occurred. The mathematical formulation of this theory on the basis of the mass law is described. The derived expressions are shown to be in accord with the experimental findings and the constants used in these expressions are shown to have definite significance. In spite of the wide variation in the properties of individual sera these expressions permit the complete description of the behavior of an unknown serum with S III without an unduly burdensome number of analyses.

The quantitative theory presented has been found applicable to other instances of the precipitin reaction, as will be shown in subsequent papers.

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Nos. 1–6 are the same as in the accompanying paper (7).

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